

AMENDMENTS TO THE SPECIFICATION

Please replace paragraphs 0039, 0047, 0048, 0055, 0058, 0065, 0069, 0071 and 0074 with the following paragraphs. Replacement paragraphs 0039, 0047, 0048, 0055, 0058, 0065, 0069, 0071 and 0074 contain markings to show changes.

[0039] FIG. 8 ~~illustrates an embodiment of the present invention including multiple electromagnetic energy detectors and an internal reference~~ shows the response of a sensor to changing glucose levels. The top line represents the change in fluorescence of the reporter group, whereas the bottom line in the graph represents the fluorescence of the reference group.

[0047] FIG. 2 is an enlargement of the optical system 2 in two typical embodiments. In FIG. 2A, a dichroic mirror or beamsplitter 11 is used to direct light from an electromagnetic energy source 7 to the optical conduit 4. Optionally, the electromagnetic energy transmitted from the energy source can be brought into a parallel beam using collimating optics, and can also be focused both before and after the beam is split (element 10). Excitation sources may consist of, but are not limited to, for example arc lamps, laser diodes, or LEDs. In this embodiment, the optical conduit 4 is a fiber optic cable, and the same fiber is used to transmit excitation light from electromagnetic energy source 7 to the sensing element 6 and also to transmit the luminescence signals from the reporter or reference groups back to the optical system 2. A dichroic element 11 preferably separates the return signal from the excitation light and directs the signal to electromagnetic energy detectors 8. Detectors may consist of, but are not limited to, for example, photodiodes, CCD chips, or photomultiplier tubes. In the event that multiple luminescent signals are returned from the sensing element, additional dichroic elements may be used to direct portions of the return signals to multiple detectors. Preferably, a luminescent reference group that is analyte insensitive is included along with the analyte-dependent reporter molecule to

provide a reference signal. This reference signal can be used, for example, to correct for optical or electronic drift.

[0048] FIG. 2B illustrates a second embodiment in which a bifurcated optical bundle or fused optical fiber arrangement is used to transmit light to and from the sensing element. Here, light from excitation source 7 is transmitted down one arm of the bifurcated fiber bundle. Optionally, the electromagnetic energy transmitted from the energy source can be brought into a parallel beam using collimating optics, and can also be focused both before and after being transmitted into the bifurcated fiber bundle (element 10). Return luminescent signals from the sensing element are detected using the second arm of the bifurcated fiber, so that in this case the fiber bundling serves to separate excitation from return luminescence. Dichroic optics, beamsplitters, or polarizers may additionally be used to further divide the return luminescence, based for example on wavelength or polarization. Optionally, bandpass filters 12 can be used to select the luminescent wavelength to be detected. Power supply 9 supplies power to the optical system 2.

[0055] The fiber used in this embodiment was a bifurcated fiber optic. It contained six 400 um fibers arranged around a central 400 um fiber. The six fibers were used as the excitation conduit and the central fiber as the detection conduit. The total diameter of the fiber was 1.4 mm. Once the fiber was polished, ~~Loctite 4011~~ LOCTITE® 4011 medical grade glue was used to adhere the sensing element to the distal end of the fiber optic. The proximal end of the fiber was bifurcated, with one arm going to an excitation source and the other arm going to a detector. A 470 nm LED was used as the excitation source, and a commercial fluorescence spectrometer was used as the electromagnetic energy detector. The emission intensity at 540 nm was then measured.

[0058] The immobilization matrix was a crosslinked alginate-based hydrogel, prepared by covalently crosslinking ~~Pronova~~ PRONOVA™ UP LVG alginate through the carboxyls with adipic acid dihydrazide (AAD) via carbodiimide chemistry. ~~Pronova~~ PRONOVA™ UP LVG

was selected in this embodiment for its low viscosity and high guluronic to mannuronic ratio. A 2% alginate solution was prepared by dissolving 1 gram of alginate in 50 mL 0.1 M MES buffer (pH 6.5) and then adding 110 mg of AAD and 79 mg of hydroxybenzotriazole (HOBt). The solution was stored at 4°C. until used. To the alginate solution, 145 mg of 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) was added per 10 mL of solution, using a dual-syringe mixing technique. The alginate, AAD, HOBt, EDC mixture was aspirated into a 1 mL syringe, and a blunt 30 gauge needle was attached to the syringe. The needle was primed, and then the tip was inserted into the catheter tubing mold on the optical fiber. The catheter tubing on the fiber was filled, ensuring good contact between the tip of the fiber optic and the alginate matrix. The matrix was allowed to cross-link for 15 minutes, and then the fiber tip and matrix assembly were transferred to a 0.1M, 6.5 pH MES solution, where they were stored for 2 hours. At the end of the two hours, the sensing tips were placed in excess phosphate buffer solution (PBS, 0.0027 M potassium chloride, 0.137 sodium chloride, pH 7.4 where they were stored a minimum of 30 minutes to quench the reaction.

[0065] An alginate matrix was then applied to the amine-functionalized fiber surface as follows. The immobilization matrix was a crosslinked alginate-based hydrogel, prepared by covalently crosslinking ~~Proneva~~PRONOVA™ UP LVG alginate, selected for its low viscosity and high guluronic to mannuronic ratio, through the carboxyls with adipic acid dihydrazide (AAD) via carbodiimide chemistry. A 2% alginate solution was prepared by dissolving 1 gram of alginate in 50 mL 0.1 M MES buffer (pH 6.5) and then adding 110 mg of AAD and 79 mg of hydroxybenzotriazole (HOBt). A 0.5 mL aliquot of this solution was then mixed with 10 mg of EDC in 50 uL of MES buffer using a dual-syringe mixing technique. The total volume of the solution was approximately 0.55 mL. The alginate, AAD, HOBt, EDC mixture was then transferred to microcentrifuge vials, and the APTES-functionalized fiber tips were submerged in the alginate solution for 3-4 minutes or until the matrix began to solidify. The tips were then removed from the alginate solution, allowed to continue reacting in air for approximately 1-10

minutes, and then transferred to 0.1 M, 6.5 pH MES buffer. The tips were allowed to sit in the MES buffer for 2 hours, and then they were quenched in excess phosphate buffer solution (PBS, 0.0027 M potassium chloride, 0.137 sodium chloride, pH 7.4) for a minimum of 30 minutes.

[0069] In another embodiment of the invention, dual wavelength detection with an internal optical reference group was performed. The binding protein was glucose galactose binding protein (GGBP), with a cysteine substituted for a glutamic acid at position 149, an arginine substituted for an alanine at position 213 and a serine substituted for leucine at position 238 (E149C/A213R/L238S). The protein was labeled at the 149 position with the reporter group N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitro- benz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD amide). The reference group was ~~Texas Red~~ TEXAS RED® C₂ maleimide attached to GGBP with a cysteine substituted for a glutamic acid at position 149 (TR-E149C GGBP). Over the physiological range of glucose concentrations, the luminescence from TR-E149C GGBP is substantially unchanged, and thus TR-E149C GGBP serves as an internal reference for the signal from the analyte-dependent binding protein and reporter group (NBD-E149C/A213R/L238S GGBP).

[0071] The immobilization matrix was a crosslinked alginate-based hydrogel, prepared by covalently crosslinking ~~Pronova~~ PRONOVA™ UP LVG alginate, selected for its low viscosity and high guluronic to mannuronic ratio, through the carboxyls with adipic acid dihydrazide (AAD) via carbodiimide chemistry. A 2% alginate solution was prepared by dissolving 1 gram of alginate in 50 mL 0.1 M MES buffer (pH 6.5) and then adding 110 mg of AAD and 79 mg of hydroxybenzotriazole (HOBt). The solution was stored at 4°C until used. Using a dual-syringe mixing technique, a 0.5 mL aliquot of the alginate solution was then mixed with a 50 uL MES solution containing 10 mg of 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) and 90 µL of 60 µM TR-E149C GGBP. The alginate, AAD, HOBt, EDC, TR-E149C mixture was aspirated

into a 1 mL syringe, and a blunt 30 gauge needle was attached to the syringe. The needle was primed, and then the tip was inserted into the catheter tubing mold on the optical fiber. The catheter tubing on the fiber was filled, ensuring good contact between the tip of the fiber optic and the alginate matrix. The matrix was allowed to cross-link for 15 minutes, and then the fiber tip and matrix assembly were transferred to a 0.1 M, 6.5 pH MES solution, where they were stored for 2 hours. At the end of the two hours, the sensing tips were placed in excess phosphate buffer solution (PBS, 0.0027 M potassium chloride, 0.137 sodium chloride, pH=7.4) where they were stored a minimum of 30 minutes to quench the reaction.

[0074] In a trial, the distal end and sensing element of a biosensor formed in this manner was inserted into solutions of PBS buffer containing different levels of glucose. Glucose levels in the solutions were measured on a clinical analyzer. FIG. 8 shows the sensor response to changing glucose levels. The 550 nm signal from the IANBD reporter group tracks changing glucose levels. The 610 nm emission from the Texas Red TEXAS RED® reporter group is substantially unchanged as glucose levels vary. However, in this embodiment, a portion of the reporter group's emission also occurs at 610 nm. The detector in the optical system that tracks the 610 nm luminescence signal detects both the emission of the reference group and also the portion of the reporter group (IANBD) emission that occurs in this wavelength region. Since the contribution to the 610 nm signal from the reporter group is a constant fraction of the 550 nm signal, this contribution can be mathematically subtracted from the 610 nm signal to generate the signal due to the reference group alone. When this mathematical manipulation is performed, the 610 nm signal is substantially unchanging with glucose concentration as shown in FIG. 8.